Aus dem Fachbereich Medizin
der Johann Wolfgang Goethe-Universität
Frankfurt am Main

betreut am
Zentrum der Biochemie
Institut für Biochemie I (Pathobiochemie)
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Translational regulation of resolution of inflammation in macrophages

Thesis
zur Erlangung des Doktorgrades der theoretischen Medizin
des Fachbereichs Medizin
der Johann Wolfgang Goethe-Universität
Frankfurt am Main

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aus Frankfurt am Main

Frankfurt am Main, 2024

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Tag der mündlichen Prüfung:	06. Juni 2024

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1. Zusammenfassung

Entzündung ist eine regulierte Reaktion des Körpers, um Gefahren wie Infektion oder Verletzung zu kontrollieren. Eine effiziente Entzündungsauflösung ist wichtig, um die Entstehung chronischer Entzündungen zu verhindern und um eine Gewebehomöostase wiederherzustellen. Makrophagen (Mϕ) spielen eine zentrale Rolle sowohl bei der Entstehung als auch bei der Auflösung von Entzündung, weil sie Pathogene sowie Gewebetrümmer phagozytieren und eliminieren. Die Efferozytose von apoptotischen Zellen ist ein wesentlicher Auslöser für die Entzündungsauflösung und trägt zu einer pro-auflösenden Reprogrammierung von Mϕ bei. Trotz der Wichtigkeit von post-transkriptionellen Regulationsmechanismen während der Auflösungsphase sowie von Translationskontrolle als wichtige Komponente für die Modulation von Genexpression in Immunzellen, bleiben relevante Translationsveränderungen in weiten Bereichen ungeklärt.

Ziel dieser Studie war die Identifizierung translationell regulierter Kandidaten in inflammatorischen primären Maus-Mϕ während der Efferozytose. Dazu habe ich eine totale RNA-Sequenzierung- sowie *de novo* Proteomik-Analysen durchgeführt, um globale transkriptionelle und translationelle Veränderungen zu analysieren. Die Sequenzierdaten bestätigten, dass Efferozytose eine pro-auflösende Signatur in inflammatorischen Mϕ induziert und deuteten auf eine Translationsregulation hin, weil die damit verbundene integrierte Stressantwort bei Efferozytose angereichert war. Während Genexpressionsveränderungen zwischen efferozytotischen und naïven Mϕ auf transkriptioneller Ebene vergleichsweise gering erschienen, beobachtete ich auf der Ebene *de novo* synthetisierter Proteine erhebliche Unterschiede. Dieser Befund deutet auf eine Translationsregulation hin. Des Weiteren wurde die enge Verbindung zwischen translationalen und metabolischen Veränderungen bestätigt, weil durch Efferozytose hochregulierte Kandidaten eine Anreicherung in Metabolismus-assoziierten Prozessen zeigten. Interessanterweise zeigte die Analyse translationell regulierter Kandidaten bei inflammatorischer Stimulation eine reduzierte Translation der meisten Kandidaten mit nur geringem Einfluss von Efferozytose. Hierunter zeichnete sich die pro-auflösende Matrix

Metallopeptidase 12 (Mmp12) als ein bisher unbekannter Kandidat ab, der zu Beginn der Entzündung translationell reprimiert und in der Auflösungsphase erhöht wurde. Der extrem hohe mRNA Gehalt sowie der nicht übermäßig hohe *de novo* Proteingehalt sind indikativ für eine potenzielle Translationsregulation von Mmp12. Validierungsexperimente rekapitulierten eine leichte Erhöhung der Mmp12 mRNA Expression und eine signifikante Reduktion des intrazellulären Proteingehalts in inflammatorischen Mϕ, übereinstimmend mit den globalen Datensätzen. Um herauszufinden, ob die Diskrepanz zwischen mRNA- und Proteinexpression auf Translationsveränderungen zurückzuführen ist, habe ich Polysomfraktionierungsanalysen durchgeführt, die eine Bestimmung des Translationsstatus von Mmp12 erlauben. Inflammatorische Mϕ zeigten eine signifikant geringere Mmp12 mRNA Abundanz in den späten Polysomen verglichen zu naïven Mϕ, wodurch auf eine reduzierte Translationseffizienz geschlossen werden kann. Folglich war der extrazelluläre MMP12 Gehalt reduziert, jedoch mit leichter Verzögerung. Die funktionelle Auswirkung verminderter Mmp12 Translation im Entzündungskontext wurde mit Migrationsexperimenten untersucht. Während siRNA-vermittelter „Knockdown" von Mmp12 die Migration auf unbeschichteten Platten nicht veränderte, erhöhte sich die Migration 3-fach auf Matrigel/Elastin-beschichteten Platten. Die Erhöhung der migrierten Distanz getrieben von siMmp12 konnte durch die Zugabe von exogenem rekombinantem MMP12 Protein gesenkt werden. Übereinstimmend mit reduzierter Mmp12 Translation und Proteingehalt in inflammatorischen Mϕ, beobachtete ich signifikant erhöhte Zellmigration auf beschichteten Platten. Daher degradiert die Mϕ Elastase MMP12 Elastin und beeinträchtigt dadurch die Migration entlang von Elastinfasern. In inflammatorischen Mϕ ist Mmp12 translationell runterreguliert, wodurch die Migrationskapazität gesteigert wird.

Zusammenfassend identifiziert diese Studie einen substanziellen Beitrag der Translationsregulation im Entzündungsverlauf, aufgezeigt durch Veränderungen zwischen inflammatorischen naïven und efferozytotischen Mϕ auf der *de novo* Proteomik Ebene. Insbesondere konnte ich die Translationsregulation von pro-auflösendem Mmp12 ermitteln, welches in früher Entzündungsphase reprimiert wird und funktionell eine erhöhte, matrix-abhängige Migration von Mϕ ermöglicht.

2. Summary

Inflammation is a regulated reaction of the body to control a threat such as infection or injury. An efficient resolution of inflammation is critical to prevent the development of chronic inflammation and to restore tissue homeostasis. Macrophages (Mϕ) play a crucial role in the onset, but also in the resolution of inflammation, because they phagocytose and eliminate pathogens and tissue debris. Efficient efferocytosis, i.e. the engulfment of apoptotic cells, represents an important trigger for the onset of the resolution response and contributes to the pro-resolving reprogramming of Mϕ. Despite the importance of post-transcriptional modes of regulation during the resolution phase and translational control as a key node modulating gene expression in immune cells, relevant translational alterations remain largely elusive.

In the present study, I aimed to identify translationally regulated targets in inflammatory primary murine Mϕ upon resolution-promoting efferocytosis. To this end, I used total RNA-sequencing as well as *de novo* proteomics analyses to determine global transcriptional and translational changes. Sequencing data confirmed that efferocytosis induced a pro-resolution signature in inflammatory Mϕ and pointed towards translational regulation because the related integrated stress response was enriched upon efferocytosis. While changes of gene expression between efferocytic and non-efferocytic Mϕ appeared rather small at the transcriptional level, I observed considerable differences at the level of *de novo* synthesized proteins. This finding suggests a regulation at the level of translation. Furthermore, the tight connection between translational and metabolic changes was confirmed by enriched metabolism-associated terms of targets upregulated by efferocytosis at both RNA and *de novo* protein level. Interestingly, analysis of translationally regulated targets in response to inflammatory stimulation showed reduced translation for most targets, with only little impact of efferocytosis. Among those targets, I identified pro-resolving matrix metallopeptidase 12 (Mmp12) as a novel candidate, which showed translational repression during early inflammation and translational increase during the resolution phase. Noteworthy, a first indicator for a potential translation regulatory component of Mmp12 were the extremely high mRNA levels and not overly high

de novo protein levels. Validation experiments recapitulated a slight elevation of Mmp12 mRNA expression and a significant downregulation of MMP12 intracellular protein levels in inflammatory Mϕ, as observed in the RNA-seq and *de novo* proteomics datasets. To investigate whether the discrepancy in mRNA and protein expression were due to changes in translation, I applied polysomal fractionation analysis to determine the translational status of Mmp12. Inflammatory Mϕ displayed a significantly lower relative Mmp12 mRNA abundance in the late polysomes compared to naïve Mϕ, suggesting reduced translational efficiency upon inflammatory stimulation. Consequently, extracellular MMP12 levels in the supernatant of inflammatory Mϕ decreased, although with a slight delay.

The functional impact of attenuated Mmp12 translation upon inflammatory stimulation was assessed in migration assays. While siRNA-mediated knockdown of Mmp12 did not alter Mϕ migration on uncoated plates, it increased migration 3-fold on matrigel/elastin-coated plates. Importantly, the increase in migrated distance driven by siMmp12 could be lowered by the addition of exogenous recombinant MMP12 protein. In line with reduced Mmp12 translation and MMP12 protein in inflammatory Mϕ, I observed a significant increase in cell migration on matrigel/elastin-coated plates, while it remained unaltered on uncoated plates. Consequently, Mϕ elastase MMP12 degrades elastin, thereby cell migration along elastin fibers is diminished. In inflammatory Mϕ, Mmp12 is translationally downregulated, thereby enhancing the migratory capacity.

In summary, the present study identifies a substantial contribution of translational regulation in the course of inflammation shown by high changes between inflammatory naïve and efferocytic Mϕ at the *de novo* proteomic level. Specifically, I was able to determine the translational regulation of pro-resolving Mmp12, which is repressed during early inflammation and recovers during the resolution phase. Functionally, translational control of MMP12 emerged as a strategy to alter the migratory properties of Mϕ, enabling enhanced, matrix-dependent migration of Mϕ during the early inflammatory phase, while restricting migration during the resolution phase.

3. Comprehensive summary

3.1 Introduction

Inflammation is a natural reaction of the body in response to injury or infection. Under homeostatic conditions, the inflammatory response is well-orchestrated and self-resolving[1]. A proper resolution of inflammation is required to restore normal tissue function, whereas an improper resolution can result in chronic inflammation that contributes to diseases such as cardiovascular diseases, cancer, and autoimmunity[2]. Macrophages (Mϕ) are important immune cells and dynamically change their metabolism and gene expression profiles during Mϕ polarization[3]. Classically activated M1 Mϕ arise after stimulation with lipopolysaccharide (LPS) and interferon γ (IFNγ), whereas alternatively activated M2 Mϕ arise after interleukin-4 (IL-4), IL-10, or IL-13 stimulation[4]. The onset of the acute inflammatory response begins with the production of soluble mediators by resident cells, e.g. tissue Mϕ, in the injured or infected tissue[5].

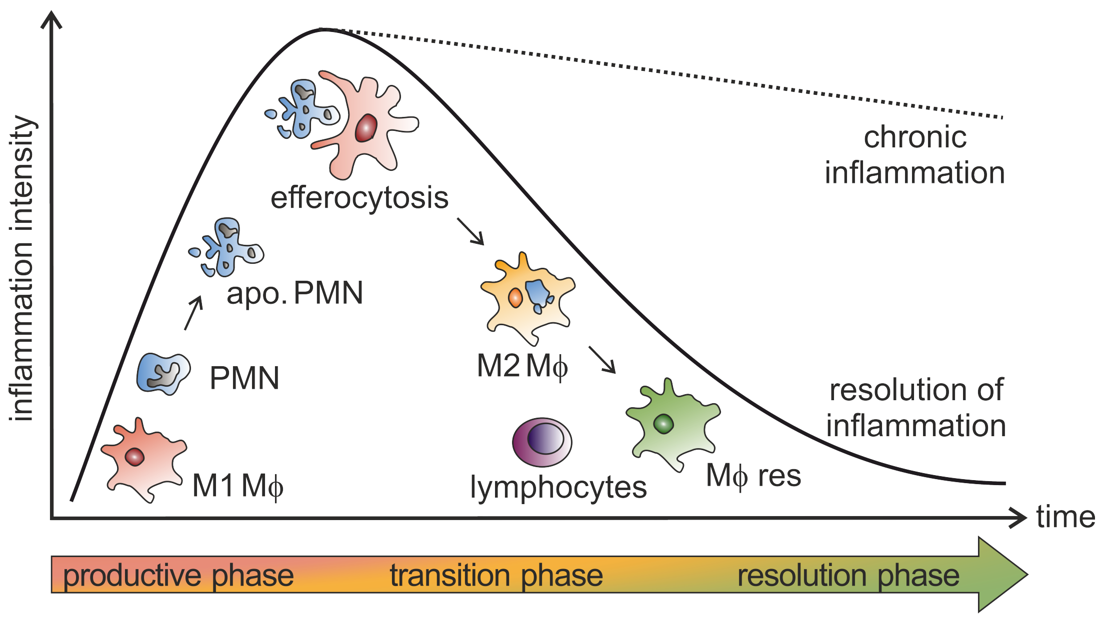

Figure 1: Course of the inflammatory response. Upon pathogen recognition, pro-inflammatory M1 Mϕ trigger the onset of an inflammatory response by releasing inflammatory cytokines, which leads to the recruitment of PMNs. In the course of the inflammatory reaction, PMNs undergo apoptosis and are cleared by Mϕ in the process of efferocytosis. During the transition phase, Mϕ undergo an efferocytosis-mediated change from M2 to resolution phase Mϕ. Infiltrating lymphocytes accompany the successful return to homeostasis. Abbreviations: (apo.) PMN: (apoptotic) polymorphonuclear neutrophils; M1 Mϕ: classically activated macrophages; M2 Mϕ: alternatively activated macrophages; Mϕ res: resolution phase macrophages.

Following pathogen recognition, Mϕ release inflammatory cytokines, for example IL-1β, IL-6, or tumor necrosis factor α (TNF-α), which are essential for the establishment of an immune response, because they facilitate cellular communication[6,7]. As a consequence, polymorphonuclear neutrophils (PMNs) are recruited to the site of inflammation, followed by an influx of monocytes that differentiate into inflammatory Mϕ[8]. PMNs release for example reactive oxygen species in order to kill infectious agents[9] and undergo apoptosis afterwards[10]. The clearance of apoptotic neutrophils by Mϕ is a process known as efferocytosis and changes the phenotype from an inflammatory to a tissue-protective, i.e. resolution phase Mϕ[11,12]. This step prevents the release of danger-associated molecular patterns (DAMPs) by secondary necrotic cells and thus represents an important trigger for the onset of the inflammatory resolution phase[13,14]. Infiltrating lymphocytes, i.e. T- and B-cells, constitute the adaptive immune response and ensure the return to homeostasis by secretion of antibodies by B-cells that neutralize the pathogen and by elimination of infected cells by activated T-cells[15]. While the onset of pro-inflammatory responses was shown to be regulated predominantly at the level of transcription, post-transcriptional modes of regulation become more important during later stages of the inflammatory process[16,17,18]. Of note, selective translational control emerged as a key mechanism in regulating gene expression in immune cells[19]. Translation describes the biosynthesis of proteins from a messenger RNA (mRNA) template and can be divided into three steps: initiation, elongation, and termination[20]. Translation initiation starts with the binding of the ternary complex, consisting of the methionine-loaded initiator transfer RNA (tRNA) bound to guanosine triphosphate (GTP)-coupled eukaryotic initiation factor 2 (eIF2) to the small (40S) ribosomal subunit. Together with eIF3 and other initiation factors, the 43S pre-initiation complex (PIC) is formed, which recognizes the mRNA by binding of eIF3 to the eIF4G subunit of the cap-binding complex. Two other important components of the cap-binding complex are eIF4E, which directly binds to the m^7G mRNA cap structure, and eIF4A, an RNA helicase unwinding RNA secondary structures[21,22,23]. eIF4G is also binding poly(A)-binding proteins (PABP), thereby circularizing the mRNA[24]. The PIC scans the 5’ untranslated region (UTR) of the mRNA in a 5’ → 3’ direction until it identifies a start codon, which results in the recruitment of the large (60S) ribosomal subunit to facilitate translation elongation[25]. The elongation step proceeds with the sequential addition of amino acids until a stop codon is reached and termination occurs, i.e. the disassembly of the small and large ribosomal subunit and the release of the synthesized polypeptide chain[26].

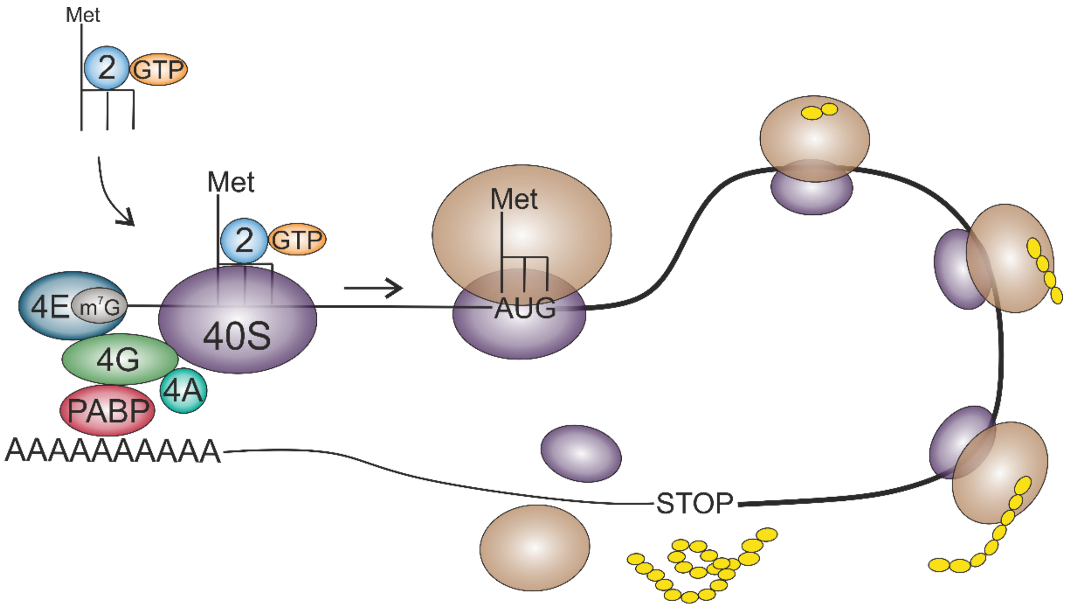

Figure 2: Schematic overview of eukaryotic translation initiation, elongation, and termination. Translation initiation starts with formation of the ternary complex consisting of GTP-coupled eIF2 bound to the methionine-loaded initiator tRNA (L-shaped symbol). Together with other eIFs, it binds the small (40S) ribosomal subunit to form the 43S PIC, which recognizes the mRNA by binding to the eIF4G subunit of the cap-binding complex. In addition, eIF4G acts as a scaffold protein facilitating the interaction with cap-binding eIF4E, the helicase eIF4A, as well as PABP. The PIC scans the 5' UTR until it encounters a start codon (AUG), resulting in the joining of the large (60S) ribosomal subunit and subsequent translation elongation. Upon encountering of a stop codon, the polypeptide chain is released and the ribosomal subunits disassemble from the mRNA. Abbreviations: eIF: eukaryotic initiation factor; GTP: guanosine triphosphate; mRNA: messenger RNA; m^7G: methyl-7-guanosine cap; PABP: poly(A)-binding proteins; PIC: pre-initiation complex; tRNA: transfer RNA; 5' UTR: 5' untranslated region.

Previous studies have shown that in IL-4-treated Mϕ translation-associated gene expression signatures were massively enriched[27]. Furthermore, when resolution was attenuated in Mϕ, translation was enriched within the down-regulated genes[28]. In addition, tumor-associated macrophages (TAMs) were found to predominantly modulate their gene expression via selective changes in translation rather than transcription during a shift from a pro- to an anti-inflammatory phenotype[29]. Although efferocytosis-induced translational changes have not been investigated before, it is well established that metabolic changes are connected to translational changes within a cell. Since translation is the most energy- and nutrient-demanding process in a cell, different nutrient environments impact translation regulation[30]. Upon cellular stresses, such as glucose or amino acid depletion, eukaryotic cells reduce global translation mediated by phosphorylation of eIF2α, which reduces the dissociation rate of eIF2B, thereby sequestering the eIF2-eIF2B complex[31,32]. On the other hand, a subset of mRNAs encoding proteins that are crucial in cell survival and stress recovery, e.g. activating transcription factor 4 (ATF4), are preferentially translated through upstream open reading frame (uORF)-mediated mechanisms[33,34,35]. This translational reprogramming under various stress conditions is achieved through the interaction of *trans*-acting regulatory factors such as RNA-binding proteins and *cis*-regulatory elements in the 5' or 3' UTR[36]. A key signaling pathway linking metabolism to translation is the mammalian target of rapamycin complex 1 (mTORC1)[37]. In case of an increased amino acid availability, phosphorylated mTORC1 promotes mRNA translation by activation of the downstream target ribosomal protein S6 kinase (RPS6K), among others[38]. On the contrary, cellular stress leads to the inhibition of mTORC1-mediated phosphorylation of 4E-binding proteins (4E-BPs). In their unphosphorylated state, 4E-BPs stay bound to eIF4E, thereby preventing its interaction with eIF4G and consequently inhibiting translation[39]. During the process of efferocytosis, Mϕ are metabolically overloaded and double their content of lipids, carbohydrates, proteins, and nucleotides[40]. However, it remains elusive which targets display translational changes and how they affect resolution of inflammation. Regulation on the level of translation enables a fast adaptation to environmental changes, which is highly relevant in the resolution of inflammation[41]. The underlying regulatory mechanism could establish starting points for new therapeutic intervention strategies.

Therefore, the aim of my study was to characterize translationally regulated targets in the context of resolution of inflammation. To this end, a murine *in vitro* efferocytosis assay was established using a co-culture of primary murine Mϕ and apoptosis-inducible cells. Novel targets regulated at the level of translation were identified by means of total RNA sequencing and *de novo* proteomics. Finally, functional consequences of translational regulation of individual targets were elucidated.

3.2 Results and discussion

Despite earlier concepts of a close correlation of mRNA and protein levels[42], there is substantial evidence of an uncoupling between the transcriptome and translatome in mammalian cells[29,43,44]. With regard to translational regulation in the context of inflammation, it was shown that in primary murine Mϕ upon infection with *Leishmania donovani*, one third of protein-coding mRNAs were differentially translated[45]. To study the largely elusive translational changes in resolution phase Mϕ, I established an *in vitro* murine efferocytosis model. Therefore, genetically modified murine NIH-3T3 caspase-8 activatable (CA) fibroblasts were co-cultured with bone-marrow derived Mϕ (BMDM). NIH-3T3 CA cells stably express a dimerizable human caspase-8 domain and thus were shown to undergo apoptotic and secondary necrotic cell death upon 6 hours dimerizer (10 nM) treatment[46].

Subsequently, I investigated the efferocytosis capacity of BMDM by measuring the green fluorescent signal emitted by the pHrodo dye upon lysosomal degradation of apoptotic cells by Mϕ[47]. I showed that Mϕ rapidly take up substantial amounts of apoptotic cells. Indeed, it has been described that the engulfment of apoptotic cells triggers the expression of efferocytic receptors, thereby resembling a positive feedback loop[48,49]. While active efferocytic processes were not detectable anymore by pHrodo assay after 16 hours of co-culture, flow cytometric analyses revealed that Mϕ still contained apoptotic cell material at this time-point. With respect to the pHrodo assay, in which the cells were excited with light every hour, it cannot be ruled out that a bleaching effect led to the decrease in green signal intensity. Therefore, I used the green cell dye carboxyfluorescein succinimidyl ester (CFSE) to label apoptotic cells before co-culturing them with red-labeled Mϕ. The high percentage of double-positive cells after overnight co-culture verifies the efferocytosis by Mϕ, although it cannot be discriminated if apoptotic cells are still actively degraded or if Mϕ contain already digested cell material. In agreement with my results, confocal microscopy experiments have confirmed double-positive BMDM after efferocytosis of *Mycobacterium tuberculosis*-infected apoptotic cells after 16 hours of co-culture[50].

In order to asses efferocytosis-mediated changes upon inflammatory stimulation of Mϕ, I co-cultured BMDM with apoptotic NIH-3T3 CA cells overnight, before stimulating the Mϕ for additional 6 or 24 hours with LPS and IFNγ. In line with previous reports[51], efferocytosis substantially reduced mRNA expression of pro-inflammatory cytokines IL-1β and IL-6 at both time-points, whereas expression of pro-resolutive IL-10 was enhanced by efferocytosis only at 6 hours of inflammatory treatment. This supports the notion that the engulfment of apoptotic cells effectively induces an inhibition of pro-inflammatory mediator release as well as modulating the macrophage phenotype towards a pro-resolution state. Hence I used this experimental setup for further analyses, as it appeared to be the optimal model system to study translational changes in the context of resolution of inflammation.

I next aimed to investigate global transcriptomic changes by performing total mRNA sequencing of untreated or inflammatory Mϕ in the presence or absence of apoptotic cells. Principal component analysis (PCA) indicated that inflammatory treatment was mainly contributing to mRNA expression changes, whereas efferocytosis accounted for only minor changes. Under inflammatory conditions, the majority of genes appeared to be rather similarly expressed between naïve and efferocytic Mϕ. Gene set enrichment analysis (GSEA) confirmed the resolution phenotype of the BMDM by enriched transforming growth factor β (TGFβ) signaling. Additionally, it showed the enrichment of the hallmark "unfolded protein response", which is a stress response of the cell that reprograms cellular translation and gene expression. Specifically, the accumulation of unfolded proteins in the endoplasmatic reticulum (ER) lead to a general suppression of translational activity through phosphorylation of eIF2α[52], whereas stress-related transcripts such as ATF4 acquire preferential translation[53,54]. Hence, global transcriptomic analyses give a first indication about the relevance of translational control in the context of the resolution of inflammation.

To asses *de novo* protein changes, i.e. translation, I performed multiplexed enhanced protein dynamics proteomics (me-PROD), which captured *de novo* synthesized peptides during the inflammatory response and therefore enables robust quantification of translation[55,56,57]. The changes between inflammatory-treated naïve and efferocytic Mϕ were much higher on the *de novo* proteomic than on the mRNA expression level, suggesting a substantial contribution of translational regulation in the course of inflammation. The tight connection between metabolic changes and translational changes was confirmed by an enrichment of autophagy- and metabolism-associated processes for targets upregulated by efferocytosis on global transcriptomic level. Additionally, at the *de novo* protein level, upregulated targets by efferocytosis were enriched for metabolism-associated terms. These findings are in accordance with previous studies suggesting that during efferocytosis, Mϕ are overloaded with apoptotic cell cargo that contributes to pro-resolving reprogramming[58]. Further support comes from the observation that mammalian target of rapamycin (mTOR) signaling, which links metabolism to translation[37,38], was enriched in mRNAs upregulated in response to efferocytosis. While translation appeared to be enriched upon efferocytosis according to the *de novo* proteomic data, this did not correspond to an increased global translation, as measured by polysome fractionation analyses. However, it needs to be considered that global translation was measured 22 hours post initial co-culture of Mϕ and apoptotic cells. As discussed above, the green signal intensity emitted by active lysosomal degradation of apoptotic cells by Mϕ in pHrodo assay was low after overnight co-culture, whereas FACS measurement showed that Mϕ still contained remainders of apoptotic cell material. It can be speculated that active engulfment and lysosomal degradation processes are completed within the first 6 hours. Therefore, it would be interesting to analyze global translation in Mϕ undergoing active apoptotic cell engulfment, i.e. after 3 hours after co-culture. Given the possibility that an overloading of Mϕ with apoptotic cell material could elicit a cellular stress response, it should be further investigated if stress-related genes show enhanced translation. Nonetheless, my data suggest a slightly reduced global translation by 6 hours of inflammatory stimulation, demonstrated by a shift from polysomes to sub-polysomes. In contrast to my findings, Schott *et al.* demonstrated a general increase in polysome association after 1 hour of inflammatory stimulation[43]. With regard to the acute inflammatory response, it is well known that transcriptomic and consequently translatomic changes occur rapidly[59]. Negative feedback mechanisms, which are activated after the initial upregulation of pro-inflammatory factors, could account for a reduction of global translation after 6 hours in comparison to the very beginning of the inflammatory response. An important observation from the *de novo* proteomic data was the translational regulation of numerous ribosomal proteins for example ribosomal protein large subunit (RPL) 14, 22, 35, and 8. This result is in line with other studies describing mRNAs, which encode proteins of the translation machinery, as primary targets of translational control under stress and their translation in a 5’ terminal oligopyrimidine (5’ TOP)-element-dependent manner[60,61].

One of my main findings was the identification of matrix metallopeptidase (Mmp12) as a target regulated specifically at the level of translation in inflammatory Mϕ. In particular, I could show the attenuated translation of Mmp12 during early inflammation. Mmp12, known as Mϕ elastase, belongs to the family of extracellular matrix (ECM) degrading enzymes[62]. Because Mmp12 has been shown to alter inflammatory responses by cleaving IFNγ[63] and to restrict leukocyte recruitment by cleaving numerous CXC chemokines[64], it was previously proposed as an inflammation resolution factor[63,65]. Although a translational regulation of Mmp12 was not reported before, studies on human Mmp13 mRNA, which is expressed in chronic inflammation, discovered translational silencing by an alternatively spliced form of the RNA-binding protein T-cell-restricted intracellular antigen-related protein (TIAR)[59]. According to my data, mRNA expression of Mmp12 remained unaltered after 6 hours of inflammatory stimulation, whereas MMP12 *de novo* peptides were significantly reduced by inflammatory stimulation in Mϕ. Total MMP12 protein expression followed the observed changes in translational output, indicating that translational changes might contribute to functional protein levels. At a later time-point of the inflammatory response, this effect was not apparent anymore. To investigate whether the discrepancy in mRNA and protein expression in inflammatory Mϕ were due to changes in translation, I applied polysome fractionation analyses to determine the translation efficiency of Mmp12 mRNA. I observed a redistribution of Mmp12 mRNA from late to early polysome fractions upon inflammatory stimulation, suggesting reduced translational efficiency. In efferocytic Mϕ, Mmp12 translation was also reduced by inflammatory stimulation, but to a lesser extent. The reduced translation not only resulted in significantly lower intracellular

MMP12 protein levels upon inflammatory stimulation, but also in reduced extracellular MMP12 levels in Mϕ supernatants, though with a minor delay. In agreement with its extracellular function, the vast majority of MMP12 was secreted. Concluding, these data point towards a translation regulatory component that accounts for the reduction of pro-resolving MMP12 protein during early inflammation.

MMPs generally act on ECM components, thereby modulating cell-matrix interactions and subsequent migration[66]. As shown earlier, MMP12 affects Mϕ recruitment in an *in vivo* model of lung inflammation[67], hence I further investigated the role of MMP12 in Mϕ migration. Therefore, I performed siRNA-mediated knockdown of Mmp12 resulting in a stable and efficient reduction of both mRNA and protein expression. Following Mϕ migration on matrigel/elastin-coated plates, Mmp12 knockdown led to a 3-fold increase of migrated distance compared to siControl transfection. Yet, Mϕ migration remained unaltered on uncoated plates, underlining the elastin substrate specificity of Mmp12. In contrast to the observed increase in Mϕ migration upon Mmp12 knockdown, studies investigating the impact of MMP proteins on three-dimensional migration mostly found a reduction of Mϕ migration upon knockdown[66]. However, the functionality of Mmp12 in two-dimensional migration, i.e. along elastin fibers, may differ substantially from the degradation of a three-dimensional matrix. In order to ensure that reduced MMP12 levels cause the increase in migration, I supplemented recombinant MMP12 protein, which abolished the increase in migration upon Mmp12 knockdown. In line with reduced MMP12 levels upon inflammatory stimulation, Mϕ increased their migratory behavior on matrigel/elastin-coated plates. Taken together, these findings suggest that Mϕ use elastin fibers as a scaffold structure for migration. Upon degradation of elastin by MMP12, the migration speed of Mϕ along the fibers is impaired. This hypothesis is supported by studies demonstrating cell migration along ECM tracks, e.g. collagen fibers[68,69].

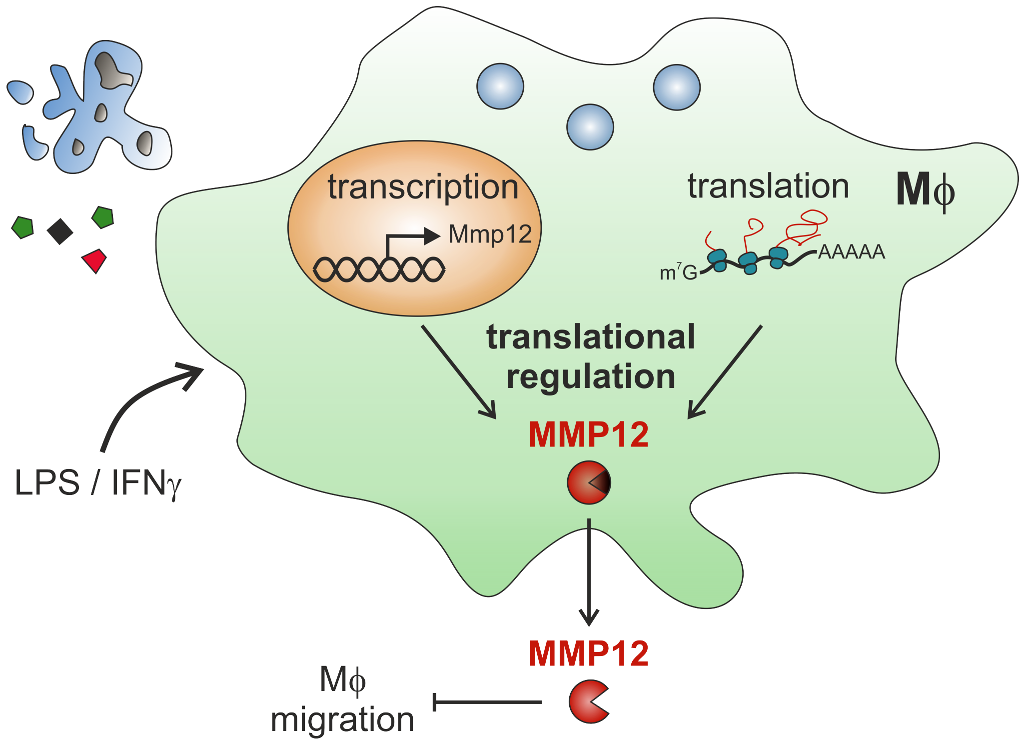

Figure 3: Model of functional implications of translational regulation of Mmp12 in the course of inflammation. Upon inflammatory stimulation of primary murine Mϕ, Mmp12 emerged as a novel translationally regulated target, i.e. showed an attenuated translation while transcription remained unaltered. Consequently, intracellular and extracellular protein levels of MMP12 were downregulated, which functionally led to an increase in Mϕ migration along elastin fibers. Abbreviations: LPS / IFNγ: lipopolysaccharide / interferon γ; Mmp12: macrophage metallopeptidase 12; Mϕ: macrophage. Adapted from Kuntschar *et al*. 2023.

The present study provides evidence for the translational regulation of Mmp12 in primary murine Mϕ during the course of inflammation. Specifically, I show that inflammatory Mϕ translationally downregulate Mmp12 during early inflammation, whereas during the resolution phase it increases again. Functionally, MMP12 appears to attenuate migration along elastin fibers. In an inflammatory environment, MMP12 levels are reduced, which increases Mϕ migration and consequently supports the mobility at the site of inflammation during the early phase of an inflammatory response.

4. Publication

International Journal of
Molecular Sciences

MDPI

Article

Mmp12 Is Translationally Regulated in Macrophages during the Course of Inflammation

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Abstract: Despite the importance of rapid adaptive responses in the course of inflammation and the notion that post-transcriptional regulation plays an important role herein, relevant translational alterations, especially during the resolution phase, remain largely elusive. In the present study, we analyzed translational changes in inflammatory bone marrow-derived macrophages upon resolution-promoting efferocytosis. Total RNA-sequencing confirmed that apoptotic cell phagocytosis induced a pro-resolution signature in LPS/IFNγ-stimulated macrophages (Mϕ). While inflammation-dependent transcriptional changes were relatively small between efferocytic and non-efferocytic Mϕ; considerable differences were observed at the level of de novo synthesized proteins. Interestingly, translationally regulated targets in response to inflammatory stimuli were mostly downregulated, with only minimal impact of efferocytosis. Amongst these targets, pro-resolving matrix metallopeptidase 12 (Mmp12) was identified as a translationally repressed candidate during early inflammation that recovered during the resolution phase. Functionally, reduced MMP12 production enhanced matrix-dependent migration of Mϕ. Conclusively, translational control of MMP12 emerged as an efficient strategy to alter the migratory properties of Mϕ throughout the inflammatory response, enabling Mϕ migration within the early inflammatory phase while restricting migration during the resolution phase.

Keywords: macrophage; inflammation; efferocytosis; Mmp12; translation; resolution

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Citation: Kuntschar, S.; Cardamone, G.; Klann, K.; Bauer, R.; Meyer, S.P.; Raue, R.; Rappl, P.; Münch, C.; Brüne, B.; Schmid, T. Mmp12 Is Translationally Regulated in Macrophages during the Course of Inflammation. *Int. J. Mol. Sci.* **2023**, *24*, 16981. https://doi.org/10.3390/ijms242316981

Academic Editor: María Martínez-Esparza

Received: 24 October 2023
Revised: 24 November 2023
Accepted: 28 November 2023
Published: 30 November 2023

1. Introduction

While inflammation is an important response to infection or injury [1], its efficient resolution is critical to prevent excessive damage due to chronic inflammatory conditions and to restore functional homeostasis [2,3]. Acute inflammatory reactions are characterized by the production of inflammatory mediators by resident cells and subsequent infiltration of polymorphonuclear cells (PMNs), primarily neutrophils, to the site of inflammation [4]. Within the highly reactive inflammatory microenvironment, PMNs undergo apoptosis and are cleared via efferocytosis by tissue-resident and recruited monocyte-derived macrophages (Mϕ) [5–7]. Their function is to phagocytose and eliminate pathogens and tissue debris. Efferocytosis further contributes to the reprogramming of macrophages from a pro-inflammatory to a pro-resolution state and, thus, represents an important trigger for initiating the inflammatory resolution phase [8–13].

While many of the acute, pro-inflammatory responses are regulated at the transcription level, post-transcriptional modes of regulation contribute to fine-tuning, gaining importance, especially during later stages of the inflammatory response [14–16]. Noteworthy, translational regulation has emerged as a key node modulating gene expression in immune cells [17]. Moreover, translation-associated gene expression signatures are massively enriched in interleukin 4 (IL-4)-treated, anti-inflammatory Mϕ [18], while the down-regulated genes are enriched in translation when resolution is attenuated in Mϕ [19]. Furthermore, changes in gene expression in tumor-associated macrophages (TAMs) during the shift from a pro- to an anti-inflammatory phenotype are predominantly modulated via selective changes in translation rather than transcription [20].

In the present study, we identified the resolution-phase associated matrix metallopeptidase 12 (Mmp12) as a novel translationally regulated target in Mϕ during inflammation and characterized the associated Mϕ migration-regulatory properties.

2. Results

While efferocytosis is critical to achieving efficient inflammation resolution, and adaptive changes during inflammation, especially during later phases, are commonly regulated at a post-transcriptional level, translational changes in Mϕ induced by efferocytosis remain largely elusive. We recently reported that translation is differentially affected when resolution is altered in a murine peritonitis model [19]. Therefore, we set out to determine the impact of efferocytosis on translational changes throughout the course of inflammation.

2.1. Efferocytosis Alters Inflammatory Responses in Macrophages

To gain insights into translational changes in Mϕ induced by efferocytosis, we initially established an in vitro murine efferocytosis model. To this end, a genetically modified murine NIH-3T3 caspase-8 activatable (CA) fibroblast cell line was used, which stably expresses a dimerizable human caspase-8 domain and, thus, was shown to undergo selective caspase-8-dependent apoptosis upon treatment with a B/B Homodimerizer (dimerizer) [21]. As expected, dimerizer (10 nM) treatment efficiently induced apoptotic and secondary necrotic cell death in CA cells. Within 6 h of adding the dimerizer, 12.1 $\pm$ 2.7% of the cells were apoptotic (Annexin V^+ PI^-), and 11.5 $\pm$ 0.9% were necrotic (Annexin V^+ PI^+) (Figure 1A, *right panel*), while after 24 h most fibroblasts appeared necrotic (64.2 $\pm$ 6.8%). In contrast, bone-marrow-derived Mϕ (BMDMs) remained unaffected by dimerizer treatment (Figure 1A, *left panel*). Since phagocytosis of apoptotic cells induces alternative Mϕ phenotypes [8], 6 h dimerizer pre-treatment was used to induce apoptosis in NIH-3T3 CA cells for subsequent co-culture with BMDMs to characterize efferocytosis-dependent changes in Mϕ translation. To determine the efferocytosis capacity of BMDMs, we labeled NIH-3T3 CA cells with pHrodo. This dye emits a green fluorescent signal only within a low pH environment, such as the lysosomal compartment of phagocytes. When co-culturing MitoTracker red-labeled Mϕ with pHrodo-labeled apoptotic cells, double-positive Mϕ were considered to actively efferocytose and lysosomally degrade apoptotic cells. Live cell imaging analyses revealed that Mϕ rapidly take up apoptotic cells, reaching maximal levels after 2 h (Figure 1B). Increasing the proportion of apoptotic cells within the co-culture from 2- to 5-fold relative to Mϕ further led to a higher uptake of apoptotic cells, which suggests that Mϕ can rapidly take up substantial amounts of apoptotic cells. Additionally, while pHrodo-labeled apoptotic CA cells alone did not emit a green signal, co-culture samples displayed a strong green signal, demonstrating the necessity of the uptake by Mϕ (Figure S1).

While efferocytosis appeared to be extremely fast, flow cytometric analyses revealed that 81.8 $\pm$ 3.7% of all Mϕ contained apoptotic cell material after overnight co-culture (Figure 1C).

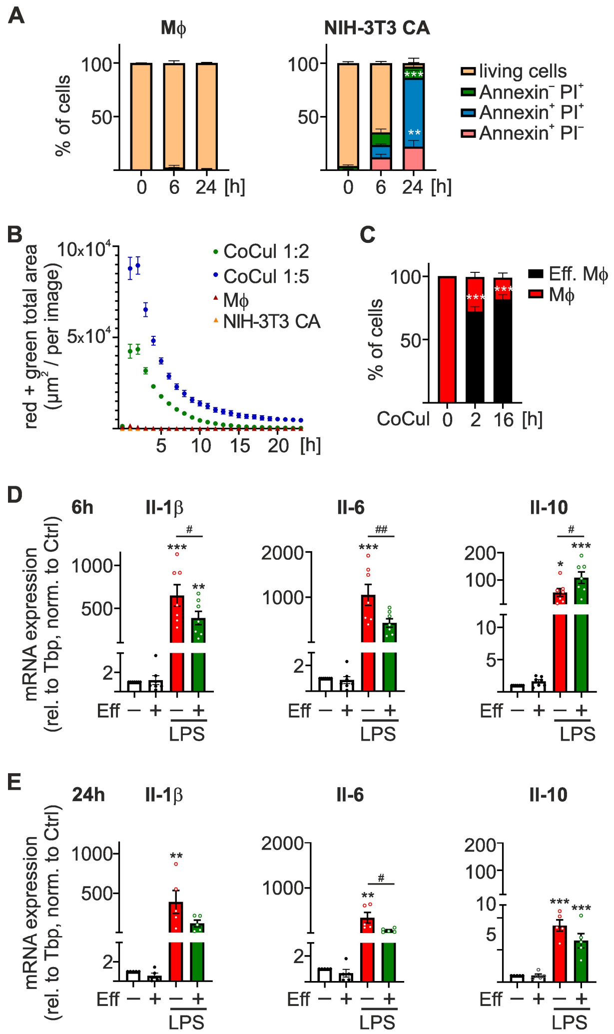

Figure 1. Impact of efferocytosis on inflammatory responses in Mϕ. (**A**) Bone marrow-derived Mϕ (BMDMs) and NIH-3T3 caspase activatable (CA) cells were treated with 10 nM dimerizer for 6 and 24 h, stained with Annexin V and propidium iodide (PI), and analyzed using flow cytometry (n = 3). (**B**) BMDMs were stained with MitoTracker red, and apoptotic NIH-3T3 CA cells (after 6 h treatment with 10 nM dimerizer) were stained with pHrodo for 1 h prior to co-culture at a 1:2 or 1:5 ratio. Efferocytosis was followed by tracking double-positive cells in an Incucyte live cell analysis system for 24 h (n = 3). (**C**) BMDMs were stained with MitoTracker red, and apoptotic NIH-3T3 CA cells (after a 6 h treatment with 10 nM dimerizer) were stained with CFSE prior to co-culture at a 1:5 ratio for 2 or 16 h. Efferocytic Mϕ were assessed by flow cytometry (n = 3). (**D**,**E**) BMDMs were co-cultured with or without apoptotic NIH-3T3 CA cells (Eff) (after 6 h dimerizer treatment) at a

1:5 ratio for 16 h, prior to stimulation with 100 ng/mL LPS and 100 U/mL IFNγ for 6 h (**D**) or 24 h (**E**) (LPS). CD45+ Mϕ were purified by magnetic-activated cell sorting (MACS) and mRNA expression was quantified by RT-qPCR analysis. mRNA expression was normalized to Tbp and is given relative to untreated control Mϕ ($n \geq 5$). Data are presented as means ± SEM and were statistically analyzed using two-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated control Mϕ; # $p < 0.05$, ## $p < 0.01$ compared to LPS/IFNγ-treated control Mϕ.

Having established the rapid efferocytosis process, which should limit the occurrence of secondary necrosis of the apoptotic cells, we next asked how efferocytosis affects Mϕ activation in our model. Therefore, we co-cultured BMDMs with 6 h dimerizer-treated NIH-3T3 CA cells at a 1:5 ratio for 16 h before stimulating washed Mϕ for an additional 6 or 24 h with LPS (100 ng/mL)/IFNγ (100 U/mL). In line with previous reports, efferocytosis markedly reduced LPS/IFNγ-induced expression of pro-inflammatory interleukin-1β (IL-1β) and IL-6 at 6 and 24 h. In contrast, efferocytosis enhanced IL-10 expression at 6 h of LPS treatment, leaving it unaltered at 24 h (Figure 1D,E). However, efferocytosis alone did not alter basal cytokine expression.

These findings suggest that efferocytosis of apoptotic cells by BMDMs might alter the kinetics of the inflammatory response to LPS/IFNγ, indicating an altered resolution phenotype.

2.2. Influence of Efferocytosis on Inflammatory mRNA Expression and Translation

To gain further insights into the impact of efferocytosis on inflammatory Mϕ responses, we performed total mRNA sequencing (RNA-seq) of untreated or 6 h LPS/IFNγ-treated Mϕ in the presence or absence of apoptotic cells. Principal component analysis (PCA) indicated that the main contributor to mRNA expression changes was LPS treatment (PC1: 77% variance), while efferocytosis appeared to account for PC2 (15% variance) (Figure S2). In total, 9589 differentially expressed genes (DEGs) (padj < 0.05) were found (Figure 2A; Table S1). While *k*-means clustering yielded a group of 1954 DEGs upregulated by efferocytosis independent of inflammatory stimulation (cluster I), no DEG group was identified as down-regulated by efferocytosis. In line with the PCA analysis, most changes appeared to be induced by LPS/IFNγ treatment. Specifically, 3163 DEGs were downregulated (cluster II), and 2867 DEGs were upregulated by inflammatory stimulation (cluster III), with fold changes similar in naïve and efferocytic Mϕ (*annotation columns*). Functionally, efferocytosis-induced DEG changes (cluster I) were strongly enriched for gene ontology (GO) terms associated with the efferocytosis process, such as autophagy and vacuole organization (Figure 2B; Table S2). In line with the close relationship between inflammation and metabolism, LPS/IFNγ-downregulated DEGs were enriched in metabolic processes, including ncRNA and DNA metabolic processes (cluster II). Meanwhile, LPS/IFNγ-induced DEGs showed a strong enrichment of immune system-associated terms (cluster III). Interestingly, although efferocytosis-induced changes contributed to a minor proportion of gene expression changes, gene set enrichment analysis (GSEA) revealed that in the inflammatory context, efferocytosis was also enriched in "TGFβ signaling", suggestive of a resolution phenotype (Figure 2C, *left panel*; Table S3). Of note, inflammatory Mϕ efferocytosis appeared to further enrich the hallmark "unfolded protein response", which alters translational processes [22].Thus, while efferocytosis induced a pro-resolution phenotype in LPS/IFNγ-stimulated Mϕ, most genes appeared to be similarly regulated between naïve and efferocytic Mϕ within the inflammatory context.

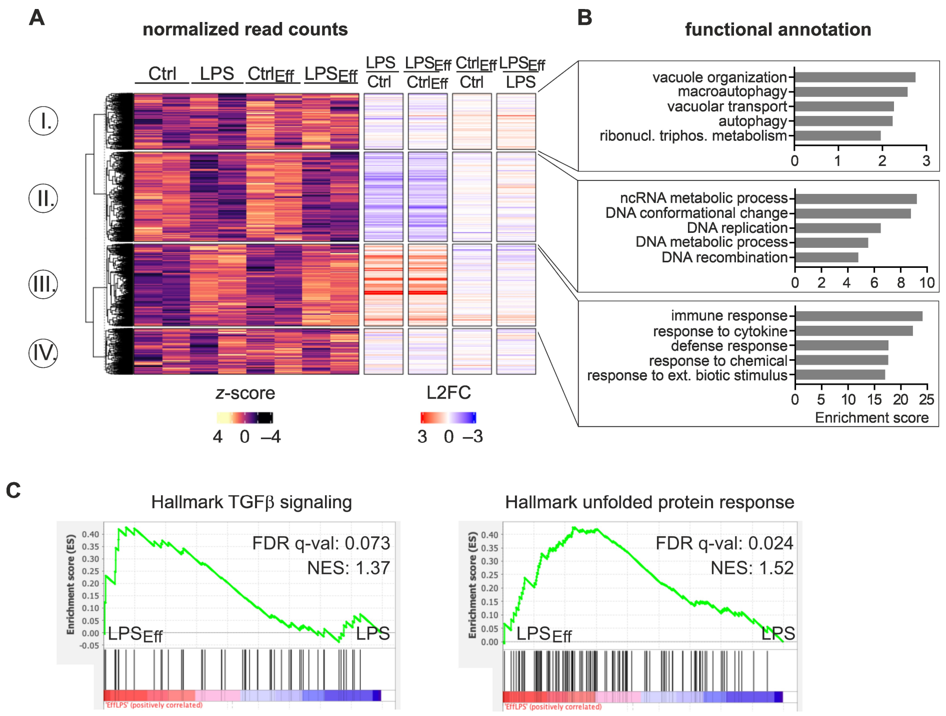

Figure 2. Differential mRNA expression changes in response to inflammatory stimulation between efferocytic and non-efferocytic Mϕ. BMDMs were co-cultured with or without apoptotic NIH-3T3 CA cells (Eff) (after 6 h dimerizer treatment) at a 1:5 ratio for 16 h prior to stimulation with 100 ng/mL LPS and 100 U/mL IFNγ for 6 h (LPS). CD45$^+$ Mϕ were purified by MACS-sorting followed by total RNA-seq analysis (n = 2). (**A**) Normalized read counts of differentially expressed genes (DEGs) (padj < 0.05) were visualized in a heatmap (z-score normalized counts) and categorized into clusters I, II, III, and IV by k-means clustering. Annotation columns depict the log2 fold change (L2FC) of DEGs. (**B**) Top five functional annotation clusters for each cluster as identified by DAVID [23,24]. (**C**) Gene set enrichment analysis (GSEA) of LPS/IFNγ-stimulated naïve vs. efferocytic Mϕ (p < 0.1, FDR < 0.1; NES = normalized enrichment score).

To determine if inflammatory responses and efferocytosis alter Mϕ functioning at a post-transcriptional level, we used multiplexed enhanced protein dynamics proteomics (mePROD) [25]. In this way, we determined changes in de novo protein expression (i.e., translation) in Mϕ induced by efferocytosis and LPS/IFNγ stimulation. k-means clustering of the 4037 differentially expressed peptides (DEPs) (padj < 0.05) identified four groups distinguishable by differential regulatory patterns (Figure 3; Table S4). First, 949 candidates were translationally enhanced by efferocytosis with minimal impact of LPS/IFNγ (cluster I) (Figure 3A). This cluster was strongly enriched for translation-associated processes (Figure 3B; Table S5). The second group of targets (1035) was enriched in ribosome- and metabolism-associated terms and displayed reduced translation in response to LPS/IFNγ but showed generally higher DEP counts in efferocytic Mϕ (cluster II). A small proportion of the DEPs (685) appeared to be induced by LPS/IFNγ treatment in naïve Mϕ with lower translational levels in efferocytic Mϕ (cluster III). This cluster was further enriched for inflammation-associated processes. While candidates in the fourth cluster appeared to be translationally repressed in efferocytic Mϕ, their translation was reduced upon inflammatory stimulation (cluster IV). Functionally, this cluster was enriched for DNA repair and cell cycle-associated functions.

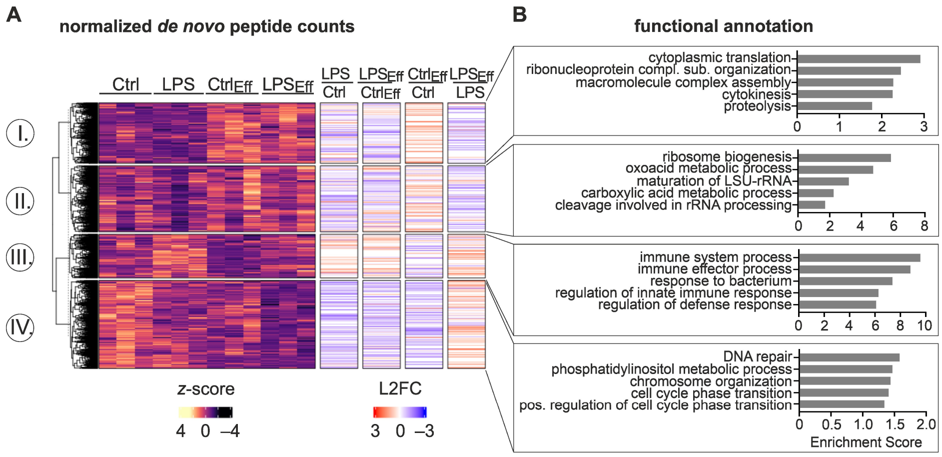

Figure 3. Differential de novo proteomic changes in response to inflammatory stimulation between efferocytic and non-efferocytic Mϕ. BMDMs were co-cultured with or without apoptotic NIH-3T3 CA cells (Eff) (after 6 h dimerizer treatment) at a 1:5 ratio for 16 h before stimulation with 100 ng/mL LPS and 100 U/mL IFNγ for 6 h (LPS). $CD45^+$ Mϕ were purified by MACS-sorting followed by multiplexed enhanced protein dynamics proteomics (mePROD) ($n = 3$). (**A**) Normalized de novo peptide counts of differentially expressed peptides (DEPs) (padj < 0.05) were visualized in a heatmap (z-score normalized counts) and categorized into clusters I, II, III, and IV by k-means clustering. Annotation columns depict log2 fold change (L2FC) of DEPs. (**B**) Top five functional annotation clusters for each cluster as identified by DAVID [23,24].

Taken together, changes between LPS/IFNγ-treated naïve and efferocytic Mϕ were much higher at the de novo proteomic (DEPs) level than at the mRNA expression (DEGs) level, suggesting a substantial contribution of translational regulation throughout the course of inflammation.

2.3. Characterization of Matrix Metallopeptidase 12 (Mmp12) Regulation throughout the Course of Inflammation

Having established global transcriptomic and de novo proteomic changes in inflammation and efferocytosis-associated resolution, we aimed to identify targets regulated specifically at the translation level during inflammation. Therefore, we selected candidates displaying strong regulation at the de novo proteomic level by LPS/IFNγ, with only minimal regulation at the mRNA expression level. Candidates with only minimal expression, as well as those responding to efferocytosis alone, were excluded. Interestingly, based on these selection criteria, we did not identify any candidate with increased translation; rather, all resulting targets showed reduced translation in Mϕ upon LPS/IFNγ stimulation (Figure 4A; Table S6). Of the resulting candidates, matrix metallopeptidase 12 (MMP12), also known as macrophage elastase, was the most highly expressed at the mRNA level without correspondingly high translation levels. The relatively low protein output, as compared to the extremely high mRNA levels, suggests a potential translation regulatory component. Notably, total MMP12 protein expression (Figure 4B, *lower panel*) corresponded with the observed translational output changes (Figure 4B, *upper panel*), suggesting that translational changes might contribute to functional protein levels.

A normalized counts

	RNA-seq				*de novo* proteomics			
	Ctrl	$Ctrl_{Eff}$	LPS	LPS_{Eff}	Ctrl	$Ctrl_{Eff}$	LPS	LPS_{Eff}
Ldha	2566	3087	2634	3814	2362	2353	1048	2559
Mmp12	**41,659**	**51,328**	**46,799**	**63,036**	**1301**	**1342**	**713**	**968**
Tpi1	1416	1428	1307	2077	1300	1528	515	1358
Sh3pxd2b	3574	4070	3957	4840	1097	1086	562	752
Pitpna	2186	2357	1932	2019	709	830	417	739
Nap1l1	467	482	396	374	477	501	338	401
Dbi	489	360	488	463	216	315	141	232
Farsa	371	429	340	369	198	226	134	158
Rps12	132	170	148	158	193	144	132	112
Ric8a	612	732	538	598	188	196	80	103

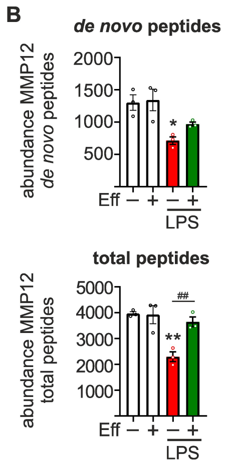

Figure 4. Selection of translationally regulated targets in inflammatory Mϕ. (**A**) For the selection of candidates predominantly regulated at the translation level upon inflammatory stimulation (100 ng/mL LPS and 100 U/mL IFNγ, 6 h), substantially expressed targets (normalized read/peptide counts > 50) were filtered for pronounced LPS/IFNγ-selective regulation of de novo proteins ($|FC_{Ctrl\ vs.\ LPS}| > 2$; $|FC_{Ctrl\ vs.\ Eff}| < 1.5$) with minimal regulation at the mRNA level ($|FC_{Ctrl\ vs.\ LPS}| < 1.2$). Normalized read (*left columns*) and de novo peptide counts (*right columns*) of the top ten selected targets, sorted by Ctrl de novo peptide counts, are shown. (**B**) Abundance of MMP12 de novo peptides (*upper panel*) and total peptides (*lower panel*) based on de novo synthesis proteomics data. Data are presented as means ± SEM and were statistically analyzed using two-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$ compared to untreated control Mϕ; ## $p < 0.01$ compared to untreated efferocytic Mϕ.

As MMP12 is important during inflammation resolution [26], we aimed to further characterize the mechanistic details of MMP12 regulation during inflammation. In line with the observations in the global data sets, Mmp12 mRNA expression remained largely unaltered (Figure 5A, *left panel*), and MMP12 protein was significantly downregulated after 6 h LPS/IFNγ stimulation, independent of the efferocytosis state (Figure 5A, *right panel*; Figure S3). In contrast, Mmp12 mRNA expression decreased 24 h after inflammatory stimulation (Figure 5B, *left panel*), while LPS/IFNγ treatment only attenuated the efferocytosis-dependent increase in MMP12 protein (Figure 5B, *right panel*; Figure S3). To investigate whether the discrepancy in mRNA and protein expression in inflammatory Mϕ was indeed due to translational changes, we used polysomal fractionation analyses to determine the translation efficiency of Mmp12 mRNA. Global translation appeared to be reduced after 6 h of LPS/IFNγ stimulation in naïve Mϕ, whereas no such effect was observed in efferocytic Mϕ (Figure 5C). While Gapdh mRNA predominantly resided in the late polysomal fractions, indicative of efficient translation, and remained unaffected by LPS/IFNγ treatment (Figure 5D, *left panel*), Mmp12 redistributed from late to early polysomal fractions, suggesting reduced translational efficiency upon inflammatory stimulation (Figure 5D, *right panel*). Interestingly, translation was also reduced in efferocytic Mϕ by LPS/IFNγ to a lesser extent, which corroborated the protein level observations (Figure 5A).

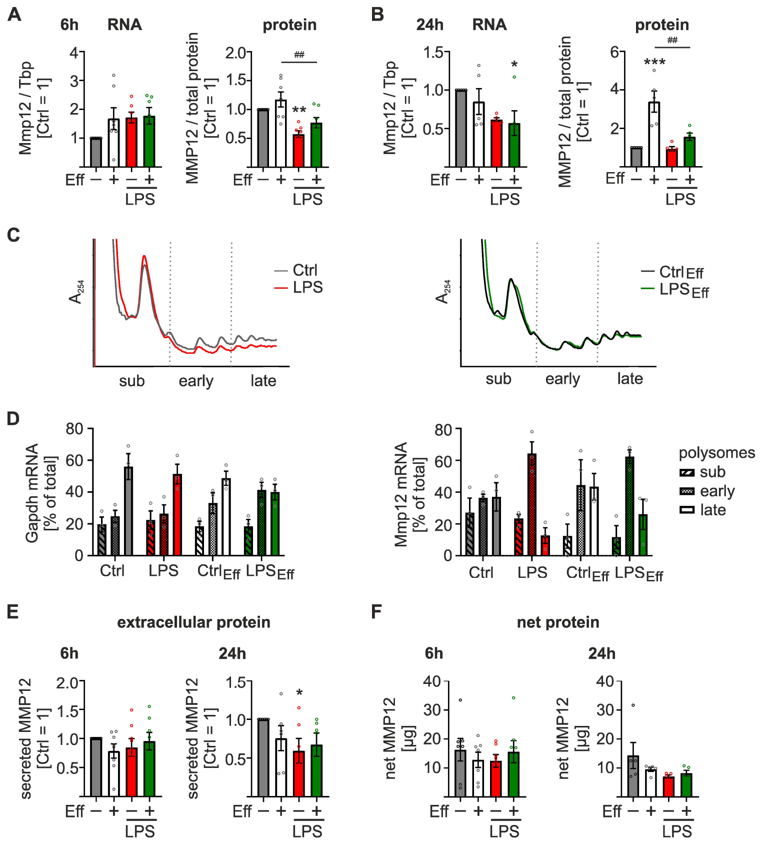

Figure 5. Translational regulation of matrix metallopeptidase 12 (Mmp12) in inflammatory Mϕ. BMDMs were co-cultured with or without apoptotic NIH-3T3 CA cells (Eff) (after 6 h dimerizer treatment) at a 1:5 ratio for 16 h prior to stimulation with 100 ng/mL LPS and 100 U/mL IFNγ for 6 or 24 h (LPS). For further analyses, CD45+ Mϕ were purified by MACS-sorting. (**A**) Mmp12 mRNA expression was quantified by RT-qPCR analysis, normalized to Tbp, and presented relative to untreated control Mϕ ($n \geq 5$). (**B**) MMP12 protein expression was analyzed by western blot analysis, normalized to total protein, and presented relative to untreated control Mϕ ($n \geq 5$). (**C**,**D**) Translational status of Mmp12 was assessed by polysomal fractionation analysis. (**C**) UV profiles identified sub-polysomal (sub), early, and late polysomal fractions (representative tracks of three independent experiments are shown). (**D**) Gapdh (*left panel*) and Mmp12 mRNA (*right panel*) distribution across the gradients was analyzed by RT-qPCR ($n = 3$). (**E**) Secreted MMP12 protein was quantified in Mϕ supernatants by ELISA and is presented relative to untreated control Mϕ ($n \geq 5$). (**F**) Net protein expression of MMP12 was calculated by combining mean intra- and extracellular MMP12 protein expression and is presented relative to untreated control Mϕ ($n \geq 5$). Data are presented as means ± SEM and were statistically analyzed using two-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated control Mϕ; ## $p < 0.01$ compared to LPS/IFNγ-treated control Mϕ.

Since MMP12 exerts extracellular functions, we assessed extracellular MMP12 protein levels in the supernatants of naïve or efferocytic Mϕ upon inflammatory stimulation. In line with the intracellular MMP12 protein levels, extracellular MMP12 levels in Mϕ supernatants decreased upon LPS/IFNγ stimulation, though with a slight delay, i.e., after 24 h (Figure 5E).

The absolute quantification of net MMP12 protein levels (i.e., the combined intra- and extracellular levels) further supported a slight decrease in MMP12 after 6 h of LPS/IFNγ treatment, which became more pronounced after 24 h (Figure 5F). As predicted based on its extracellular function, most MMP12 (99.99972% +/− 0.00024%) appeared to be secreted in all samples, independent of the treatments.

In summary, these data suggest that the reduction in pro-resolving MMP12 protein during early inflammation is largely controlled translationally.

2.4. MMP12 Suppresses Migration of Macrophages

As MMP12 has been suggested to affect macrophage recruitment in an in vivo model of lung inflammation [27], we hypothesized that its expression might play a role in BMDM migration. To assess the impact of MMP12 on migration, we transfected Mϕ with siRNA targeting Mmp12 (siMmp12). This efficiently and stably reduced Mmp12 expression between 24 and 72 h after transfection at the mRNA and protein levels (Figure 6A). Unexpectedly, Mmp12 knockdown increased Mϕ migration on Matrigel/elastin-coated plates by approximately 3-fold compared to siControl-transfected Mϕ (Figure 6B), without altering Mϕ migration on uncoated plates (Figure S4A).

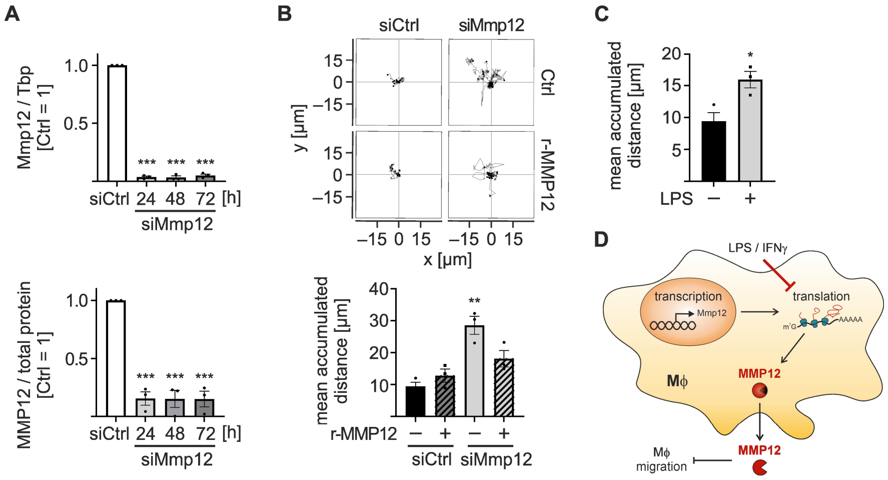

Figure 6. Impact of MMP12 on Mϕ migration. BMDMs were transfected with Mmp12 or Ctrl siRNA (50 nM) for 24, 48, or 72 h. (**A**) Mmp12 mRNA expression was quantified by RT-qPCR analysis, normalized to Tbp, and presented relative to siCtrl-transfected Mϕ (72 h) (*n* = 3; *upper panel*). MMP12 protein expression was analyzed by Western blot analysis, normalized to total protein stain, and presented relative to siCtrl-transfected Mϕ (72 h) (*n* = 3; *lower panel*). (**B**,**C**) Mϕ were seeded 48 h after transfection on Matrigel (0.5×)/elastin (50 µg/mL) coated plates. (**B**) Mϕ were treated with recombinant MMP12 (r-MMP12, 50 ng/mL) 24 h after seeding, and migration was determined by live cell tracking for 24 h and quantified using the ImageJ manual tracking plugin. Representative tracks of siCtrl and siMmp12 in the presence or absence of r-MMP12 are depicted (*upper panel*). The migrated distance of 20 randomly selected cells per field of view was analyzed per replicate (*n* = 3;

lower panel). (**C**) siCtrl-transfected Mϕ were stimulated with 100 ng/mL LPS and 100 U/mL IFNγ (LPS) 24 h after seeding, and migration was determined by live cell tracking for 24 h (n = 3). Data are presented as means ± SEM and were statistically analyzed using two-way ANOVA with Tukey's multiple comparisons test (**A**,**B**) or unpaired t-test (**C**); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated, siCtrl-transfected Mϕ. (**D**) Schematic model of the translational repression of MMP12 in Mϕ by pro-inflammatory LPS/IFNγ stimulation, resulting in reduced MMP12 protein levels and enhanced Mϕ migration in the local environment.

To ensure that the increase in migration was due to reduced MMP12 levels, we supplemented recombinant MMP12 protein (r-MMP12), which efficiently reduced migration in Mmp12-knockdown Mϕ without affecting control Mϕ (Figure 6B). Again, this effect was only observed on Matrigel/elastin-coated plates, not on uncoated plates (Figure S4B). To assess if LPS/IFNγ treatment, which efficiently reduced Mmp12 translation and MMP12 protein, also affects Mϕ migration, we evaluated Mϕ migration in the presence of LPS/IFNγ for 24 h. In line with the reduced MMP12 levels in inflammatory Mϕ, migration specifically increased only on Matrigel/elastin-coated plates (Figure 6C), while migration on uncoated plates remained unaltered (Figure S4C).

Taken together, these findings suggest that the Mϕ elastase MMP12 degrades elastin and thereby prevents the migration of Mϕ along elastin fibers. In an inflammatory context, translational downregulation of MMP12 enhances the migratory capacity of Mϕ, supporting the mobility of Mϕ at the site of inflammation during the early phase of the inflammatory process.

3. Discussion

In the present study, we analyzed transcriptional and translational changes in Mϕ upon stimulation with LPS/IFNγ. Further, we assessed changes in the inflammatory RNA and protein expression profiles in Mϕ brought about by the efferocytosis of apoptotic cells. While efferocytosis induced resolution-related transforming growth factor β (TGFβ) signaling and translation-regulatory gene expression signatures in inflammatory Mϕ, we found that the observed transcriptional changes were determined largely by inflammatory stimuli. In contrast, efferocytosis substantially altered de novo synthesized proteins in naïve and inflammatory Mϕ. We further observed a marked, exclusively translational repression of the resolution-associated Mϕ elastase Mmp12 during early inflammation, resulting in a delayed reduction in functional extracellular MMP12 protein levels. Functionally, reduced Mmp12 expression in Mϕ, e.g., during inflammation, enhanced their migratory capacity (Figure 6D).

In contrast to earlier concepts suggesting that mRNA and protein levels are generally closely correlated [28], there is accumulating evidence that transcriptome and translatome changes in response to stimulation diverge substantially in mammalian cells [20,29,30]. For instance, upon infection of primary murine Mϕ with *Leishmania donovani*, a third of the protein-coding mRNAs was differentially translated [31]. Moreover, in tumor-associated Mϕ, gene expression changes during tumor outgrowth appeared to be regulated predominantly via translational regulation [20]. Similarly, we observed marked differences in de novo proteomic changes between the inflammatory response of efferocytic and non-efferocytic macrophages. At the transcriptomic level, regulatory patterns appeared rather similar, suggesting a major contribution of translational changes to the efferocytosis-dependent modulation of inflammatory responses in Mϕ. While efferocytosis-induced translational changes have not been investigated, the close relationship between metabolic and translational changes is well established. For example, glucose or amino acid depletion elicits a stress reaction, which enhances phosphorylation and, thus, inactivates eIF2α, consequently reducing global translation [32,33], while favoring the synthesis of a specific subset of stress response factors such as activating transcription factor 4 (ATF4) [34]. Similarly, targets upregulated by efferocytosis at the RNA level were enriched in autophagy- and metabolism-associated processes, and those upregulated at the de novo protein level were enriched in metabolism-associated terms (Tables S2 and S5). These findings corroborate previous reports suggesting that Mϕ are metabolically overloaded and double their lipid, carbohydrate, protein, and nucleotide content [10,35]. Surprisingly, while translation also appeared to be enriched upon efferocytosis at the de novo proteomic level, this did not correspond to an increase in global translation, as measured via polysomal fractionation analyses. Yet, other studies investigating translational regulation during inflammation also observed a pronounced enrichment in translation-associated candidates, within the translationally regulated targets [36]. Accordingly, we observed translational regulation of numerous ribosomal proteins, such as RPL14, 22, 35, and 8, all known to be translated in a 5′ terminal oligopyrimidine (5′ TOP) element-dependent manner [37]. Further support for the impact of metabolic changes during efferocytosis on translation comes from the observation that mammalian target of rapamycin (mTOR) signaling was enriched in mRNAs upregulated in response to efferocytosis (Tables S2 and S3). mTOR is well established as a crucial signaling node linking metabolism to translation [38,39].

As a side note, the impact of translational regulation on the functional net protein output is likely underestimated in many studies, as transcription and translation are commonly regulated in the same direction. Yet, even in the case of co-directional regulation, translation provides a last control measure to tune expression.

Mmp12 is induced by granulocyte macrophage-colony stimulating factor (GM-CSF) and further enhanced by co-stimulation with TGFβ, IL-1β, and monocyte chemoattractive protein 1 (MCP-1). Additionally, activation of CD40 signaling or anti-inflammatory stimulation with IL-4 induces, while IFNγ attenuates Mmp12 expression [40–42]. Here, we observed that while Mmp12 mRNA expression was not affected by LPS/IFNγ, Mmp12 translation was markedly reduced in Mϕ during the early stages of inflammation, reducing levels of secreted functional MMP12 at later time points. While translational regulation of Mmp12 was not previously reported, human Mmp13 mRNA, which is expressed in chronic inflammation, was translationally silenced by an alternatively spliced form of the RNA-binding protein TIAR (T-cell-restricted intracellular antigen-related protein) [43]. Hence, it will be interesting to determine whether a similar mechanism regulates Mmp12 translation or if other RNA-binding proteins are involved. Nevertheless, in line with the stimulatory function of CD40 [40] and the inhibitory function of IFNγ [41], negative regulation of CD40 signaling as well as positive regulation of IFNγ signaling were enriched in LPS/IFNγ-induced genes (Figure S5). Notably, other MMPs, such as Mmp9 and Mmp13, appeared to be induced exclusively by pro-inflammatory stimuli, such as LPS, IL-1β, or TNF [44].

Functionally, MMP12, known as macrophage elastase, belongs to the family of extracellular matrix (ECM) degrading enzymes [45]. It was further proposed as an inflammation resolution factor [46] and shown to alter inflammatory responses by cleaving IFNγ [26], but also via restricting recruitment of leukocytes by cleaving numerous CXC chemokines [47]. However, MMPs also generally act on ECM components, altering cell-matrix interactions and migration [48]. While MMP12 was previously shown to be critical for Mϕ transmigration across intestinal epithelial cell layers in severe colitis [49], we found that MMP12 attenuates Mϕ mobility on Matrigel/elastin-coated surfaces resembling structural features of the ECM [50]. Specifically, reduced Mmp12 expression, achieved via knockdown or inflammatory stimulation, enhanced the Mϕ migratory capacity, which could be overcome by adding exogenous MMP12. The fact that this altered migration phenotype was only observed in the presence of elastin (i.e., the substrate of MMP12) in the scaffold suggests that Mϕ might use elastin fibers as tracks. In fact, collagen fibers were recently proposed as ECM tracks supporting cell migration [51], and nanofiber-based, specific reconstructions of ECM fibers revealed that cell migration properties depend on the exact composition of the fibers [52].

4. Conclusions

We provide the first evidence that Mmp12 can be regulated at the translation level. Specifically, we show that Mmp12 is translationally repressed during early inflammation in primary murine Mϕ and increases during the resolution phase. Since MMP12 appears to attenuate migration along elastin fibers, reduced MMP12 levels in an inflammatory environment might enhance the ability of Mϕ to patrol the site of inflammation while simultaneously limiting their trans-epithelial egress. Thus, increasing MMP12 levels at later stages of the inflammatory process might allow for the emigration of macrophages, contributing to the normalization of the local immune cell environment.

5. Materials and Methods

5.1. Chemicals

All chemicals were obtained from Thermo Fisher Scientific (Dreieich, Germany) unless otherwise indicated. B/B Homodimerizer (dimerizer) was purchased from Takara Bio Europe (Saint-Germain-en-Laye, France), and recombinant murine MMP12 from R&D Systems (Minneapolis, MN, USA).

5.2. Cell Culture

Bone marrow-derived Mϕ (BMDMs) were isolated from the femurs of adult male and female C57BL/6 mice (>8 weeks) and differentiated with 20 ng/mL macrophage colony-stimulating factor (M-CSF) and 20 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF) (both from Immunotools, Friesoythe, Germany) in Dulbecco's modified eagle medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS; Capricorn Scientific, Ebsdorfergrund, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin (both from Thermo Fisher Scientific) over 6 days. NIH-3T3 caspase activatable (CA) cells (clone D10) (kindly provided by Prof. Simone Fulda, Frankfurt, Germany) were cultured in DMEM supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

5.3. Efferocytosis Model

NIH-3T3 CA cells were stimulated for 6 h with the dimerizer (10 nM) to induce apoptosis before adding them to differentiated BMDMs at a 1:5 ratio for 16 h in the continued presence of the dimerizer (10 nM). For inflammatory stimulation, apoptotic cells were removed, BMDMs were washed twice with PBS and then stimulated for 6 h with 100 ng/mL LPS and 100 U/mL murine IFNγ (both from Sigma-Aldrich, Taufkirchen, Germany). $CD45^+$ cells were isolated with CD45 MicroBeads by MACS-sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions for further analyses.

5.4. Viability Assay

The viability of NIH-3T3 CA cells and BMDMs was determined by Annexin V (Immunotools) and propidium iodide (PI; Thermo Fisher Scientific) staining and subsequent fluorescence-activated cell sorting (FACS) analysis on a FACSymphony A5 instrument (BD Biosciences, Heidelberg, Germany).

5.5. Efferocytosis Assays

To determine efferocytic capacity, apoptotic NIH-3T3 CA cells were stained with pHrodo green (Thermo Fisher Scientific) 6 h after dimerizer (10 nM) treatment, and Mϕ were stained with MitoTracker red. One hour after staining, pHrodo-labeled apoptotic cells were added to MitoTracker-labeled Mϕ at 2:1 and 5:1 ratios. Efferocytic Mϕ were identified as the double-positive cells on an Incucyte® S3 live cell analysis system (Sartorius, Göttingen, Germany).

Alternatively, apoptotic cells were stained with the CFSE Cell Division Tracker kit (Biolegend, San Diego, CA, USA) prior to co-culture with MitoTracker-labeled Mϕ at a 5:1 ratio; efferocytic Mϕ were identified as the double positive cells on a FACSymphony A5 instrument.

5.6. RNA Isolation, Reverse Transcription, and Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was isolated using TRIzol according to the manufacturer's instructions. RNA was reverse transcribed with the Maxima First Strand cDNA Synthesis Kit, and qPCR analyses were performed using PowerUp SYBR Green Master Mix on QuantStudio 3 and 5 PCR Real-Time Systems (Thermo Fisher Scientific). Primers were obtained from Biomers (Ulm, Germany) and are listed in Table S7.

5.7. Western Blot Analysis and ELISA

All reagents used for western blotting were purchased from Sigma-Aldrich unless otherwise indicated. Cells were lysed in lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40; freshly supplemented with 1 mM DTT from Carl Roth, Karlsruhe, Germany; protease inhibitor and phosphatase inhibitor mixes from cOmplete and phosSTOP; Roche, Grenzach-Wyhlen, Germany). Next, 50 µg of total protein was separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (GE Healthcare, Chalfont St Giles, UK). Proteins were detected using a specific antibody for murine MMP12 (R&D Systems, Minneapolis, MN, USA) and appropriate IRDye secondary antibodies (LI-COR Biosciences, Bad Homburg, Germany) and quantified on an Odyssey infrared imaging system (LI-COR Biosciences).

Extracellular quantities of MMP12 were measured in cell culture supernatants using the mouse MMP12 ELISA kit (Abcam, Cambridge, UK) according to the manufacturer's instructions on an absorption microplate reader (Berthold Technologies, Bad Wildbad, Germany).

5.8. Polysomal Fractionation

BMDMs were subjected to polysomal fractionation as described previously [53]. Briefly, cells were incubated with 100 µg/mL cycloheximide (CHX, Carl Roth) for 10 min, washed with PBS/CHX (100 µg/mL), and lysed in 750 µL polysome lysis buffer (140 mM KCl, 20 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.5% NP-40, 0.5 mg/mL heparin, 1 mM DTT, 100 U/mL RNasin from Promega, Mannheim, Germany; 100 µg/mL CHX). After pelleting, 600 µL of the cell lysates were layered onto 11 mL of 10–50% continuous sucrose gradients; 100 µL of the lysate was used for total RNA isolation. Gradients were centrifuged at 35,000 rpm for 2 h at 4 °C without brake using a SW40 rotor (Beckman Coulter, Brea, CA, USA). The gradient was collected into 1-mL fractions using a Gradient Station (BioComp Instruments, Fredericton, Canada), and UV absorbance was measured at 254 nm. RNA was precipitated by adding 1/10 volume of sodium acetate (3 M) and 1 volume of isopropyl alcohol. RNA was further purified using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and pooled into sub-polysomes (fractions 1–4), early polysomes (fractions 5–7), and late polysomes (fractions 8–11). Finally, 500 ng of RNA was reverse-transcribed and quantified by qPCR as described above.

5.9. RNA Sequencing

RNA was isolated using the RNA Clean & Concentrator-25 kit (Zymo Research, Freiburg, Germany), and rRNA was depleted from total RNA using the RiboCop rRNA depletion kit (Lexogen, Vienna, Austria) according to the manufacturer's instructions. RNA quality and quantity were determined using RNA ScreenTape assays on a TapeStation 4150 (Agilent Technologies, Waldbronn, Germany) and Qubit RNA HS Assay Kits on a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). Sequencing libraries were prepared according to the total RNA workflow of the TruSeq Ribo Profile (Mammalian) Library Prep Kit (Illumina, San Diego, CA, USA). Briefly, rRNA-depleted RNA was heat-fragmented (94 °C, 25 min) and end-repaired. Thereafter, the 3′ adapter was ligated, and reverse transcription was performed. After PAGE purification, cDNA was circularized, and PCR amplified. The quality of cDNA libraries was assessed using HS-D1000 ScreenTape assays on a TapeStation 4150, and quantities were measured using Qubit dsDNA HS Assay Kits. Libraries were sequenced (single end, 50 cycles) using a P2 100-cycle kit on a NextSeq 2000 instrument (Illumina).

Data processing was performed using cutadapt for adapter trimming [54], bowtie2 for rRNA removal [55], and STAR for mapping the samples to the mouse genome (mm39) [56]. Transcript counts were determined using htseq-count with default parameters [57]. Differentially expressed genes were determined using DESeq2 in R [58]. *P* values were adjusted by Benjamini-Hochberg FDR correction. Differentially regulated transcripts were visualized with ComplexHeatmaps R package [59] by subjecting read counts to row-wise *z*-score normalization and grouping by *k*-means clustering. To identify enriched functional annotation clusters, transcripts were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) against the gene set "GOTERM_BP_ALL" [23,24]. A list of all detected transcripts (basemean > 0, for all conditions) served as the background data set.

5.10. De Novo Proteomics (Multiplexed Enhanced Protein Dynamics (mePROD) Proteomics)

For pulse labeling experiments, BMDMs were co-cultured with apoptotic NIH-3T3 CA cells, as described before. After washing two times with PBS, cells were incubated with DMEM medium for SILAC containing 100 µg/mL Arg10 and 100 µg/mL Lys8 (both from Cambridge Isotope Laboratories, Tewksbury, MA, USA) and stimulated with 100 ng/mL LPS and 100 U/mL IFNγ for 6 h. To obtain a fully labeled sample, BMDMs were cultured in DMEM for SILAC for two weeks. For cell harvest, $CD45^+$ cells were isolated out of co-cultures as described before. A fully labeled sample and an unlabeled sample were washed three times with PBS. After pelleting, 1×10^6 cells were flash-frozen in liquid nitrogen. Sample preparation for LC-MS, mass spectrometry and subsequent data analysis and statistics were performed as described previously [25,60,61].

5.11. Migration Assay

Differentiated BMDMs were transfected using HiPerFect transfection reagent (Qiagen) with 50 nM Mmp12 (ON-TARGET plus Mouse Mmp12 siRNA—SMARTpool) or Ctrl siRNA (ON-TARGETplus Non-targeting Pool; both Dharmacon, Lafayette, LA, USA) for 48 h and seeded in 24-well culture plates coated with $0.5 \times$ Matrigel supplemented with 50 µg/mL mouse elastin (Sigma-Aldrich). After 24 h, the cells were imaged every 10 min for 24 h at 37 °C and 5% CO_2 using a Cell Observer microscope (Carl Zeiss, Oberkochen, Germany). Analysis of Mϕ migration was performed using the ImageJ (v. 2.0.0-rc-56) manual tracking plugin. Twenty randomly chosen cells per field of view were tracked per condition for each replicate. Migration plots were generated for 20 tracks using the Chemotaxis and Migration Tool from ibidi (Gräfelfing, Germany).

5.12. Statistical Analysis

Statistics were performed with GraphPad Prism v9.3.1 (GraphPad Software, San Diego, CA, USA). Data are reported as means ± SEM of at least three independent experiments. Normal distribution was assessed using the Shapiro-Wilk test. Statistically significant differences were calculated using two-way ANOVA with Tukey's multiple comparisons test or Students *t*-test.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms242316981/s1.

Author Contributions: Conceptualization, S.K., B.B. and T.S.; formal analysis, S.K., K.K., C.M. and T.S.; funding acquisition, B.B. and T.S.; investigation, S.K., G.C., K.K., R.B., S.P.M., R.R. and P.R.; methodology, S.K. and K.K.; resources, B.B.; supervision, T.S.; writing—original draft, S.K. and T.S.; writing—review & editing, S.K., G.C., R.B., S.P.M., R.R., P.R., C.M., B.B. and T.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Deutsche Forschungsgemeinschaft (GRK 2336, TP6; SCHM2663/7-1). The APC was funded by the Open Access Publication Fund of Goethe University.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequencing data presented in this study are available in GEO accession number GSE245834.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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5. List of publications

1. **Kuntschar S**, Cardamone G, Klann K, Bauer R, Meyer SP, Raue R, Rappl P, Münch C, Brüne B, Schmid T. Mmp12 Is Translationally Regulated in Macrophages during the Course of Inflammation. *Int. J. Mol. Sci.* 2023;24:16981. doi: 10.3390/ijms242316981

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6. Contribution statement on publications

1. I conceived the study and experimental design, performed and analyzed the experiments, conducted the bioinformatic analysis, and wrote the original draft of the manuscript.

2. As a co-author, I supported molecular biology analyses and isolation of primary cells.

3. As a co-author, I supported molecular biology analyses.

4. As a co-author, I supported the next-generation sequencing experiments.

5. As a co-author, I supported the polysome profiling experiments.

6. As a co-author, I supported molecular biology analyses and isolation of primary cells.

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Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Prüfung eingereichte Thesis mit dem Titel

Translational regulation of resolution of inflammation in macrophages

im Zentrum der Biochemie, Institut für Biochemie I (Pathobiochemie) unter Betreuung und Anleitung von PD Dr. Tobias Schmid ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Thesis angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Thesis oder Dissertation eingereicht.

Die Grundsätze der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Sicherung guter wissenschaftlicher Praxis in ihrer gültigen Form liegen mir vor und wurden bei der wissenschaftlichen Arbeit eingehalten.

Vorliegende Ergebnisse der Arbeit wurden in folgendem Publikationsorgan veröffentlicht:

Kuntschar S, Cardamone G, Klann K, Bauer R, Meyer SP, Raue R, Rappl P, Münch C, Brüne B, Schmid T. Mmp12 Is Translationally Regulated in Macrophages during the Course of Inflammation. Int. J. Mol. Sci., 24, 16981, 2023

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(Ort, Datum) (Unterschrift)